

Population genetics and systematics of Asian elephant (*Elephas maximus*): A study based on sequence variation at the Cyt b gene of PCR-amplified mitochondrial DNA from hair bulbs

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Abstract

To investigate genetic variability and differentiation in Asian elephant (*Elephas maximus*) a total of 53 individuals from Sri Lanka, southern India, northeastern India, northern and southern Myanmar (Burma), northern and eastern Thailand, and Vietnam were screened for sequence variation at the mitochondrial cytochrome b gene. An about 480 bp part of the gene was PCR amplified from hair bulbs, directly cycle-sequenced, and 335 bp were analysed on a direct blot sequencing device. Eight haplotypes, differing from one another by one to six third position transitions at a total of seven polymorphic sites, were found. They differed from published sequences of African elephant (*Loxodonta africana*) by at least 18, and from those of the Siberian woolly mammoth (*Mammuthus primigenius*) by at least eight nucleotide substitutions. Haplotypes separated out by nucleotide divergence into two major groups, which showed differences in their geographic distribution. However, there was no indication of a major separation between elephants from Sri Lanka and the Asian mainland, previously assumed to represent two different subspecies. Populations studied showed remarkable differences as to estimates of haplotype and nucleotide diversity. Phylogeographic patterns of haplotypes suggest that Asian elephants have formed a coherent population after their Pliocene invasion of southern and southeastern Asia. Taking into account records on population histories, the present local differentiation can be explained by varying human impact on population structure and size.

Introduction

Asian elephant (*Elephas maximus*) once was broadly distributed from Persia through the Indian subcontinent into southern and southeastern Asia, and into China up to the Yangtse Kiang. However, as a consequence of habitat destruction, hunting, poaching and capturing by man, this species has experienced a rapid and dramatical decline in population numbers (SUKUMAR 1990; KURT et al. 1995). Even though the grand total of elephants in Asia still ranges from 34,000 to 56,000 (SUKUMAR 1990), given the subdivision of populations into several large matrilinear family clans and the fragmentation of habitats, estimates of the respective genetically effective population size (N_e) do not exceed about 1,000 in the best case (Sri Lanka, see MCKAY 1973).

Genetic management of both wild and captive populations of Asian elephant requires knowledge on levels of genetic diversity within and among populations, and on systematic relationships among populations living in different parts of southern and southeastern Asia. Both demands are currently only scarcely fulfilled. Population genetic data avail-

able to date are based on protein electrophoresis, carried out in a small number of individuals, and give a quite contradictory picture as to levels of polymorphism and heterozygosity (DRYSDALE and FLORKIEWICZ 1989; NOZAWA and SHOTAKE 1990; HARTL et al. 1995). Also the systematics of Asian elephant is far from being settled. Three subspecies – *E. maximus maximus* on Sri Lanka, *E. m. indicus* in India and Indochina, and *E. m. sumatranus* on Sumatra – are presumed to exist (HAYNES 1991), but electrophoretic evidence as to a major separation between elephants from Sri Lanka and from India (elephants from Sumatra were not studied) proved to be inconsistent (NOZAWA and SHOTAKE 1990; HARTL et al. 1995).

The present study aims at characterizing genetic diversity in Asian elephant as well as differentiation among populations from Sri Lanka, India, Myanmar (Burma), Thailand, and Vietnam on the basis of cytochrome b (Cyt b) sequences of mitochondrial DNA, PCR-amplified from hair bulbs.

Material and methods

Sample collection

Hair samples were collected from a total of 53 Asian elephants living in southern Asian elephant camps or European zoos. Specimens originated from southern Sri Lanka ($n = 2$), eastern/northeastern Sri Lanka ($n = 12$), southern India (Kerala, $n = 9$), northeastern India ($n = 3$), northern Myanmar ($n = 8$), southern Myanmar ($n = 3$), northern Thailand ($n = 5$), eastern Thailand ($n = 6$), and Vietnam ($n = 5$) (Fig. 1). To achieve larger sample sizes, five study units were formed: 1. Sri Lanka with a continuous elephant population; 2. Southern India, isolated by the Arabian Sea towards the south and by extensive forest clearings towards the north; 3. Northeastern India and northern Myanmar, where there is a large, more or less continuous population in Assam and Nagaland on the Indian side, and Myitkyina and

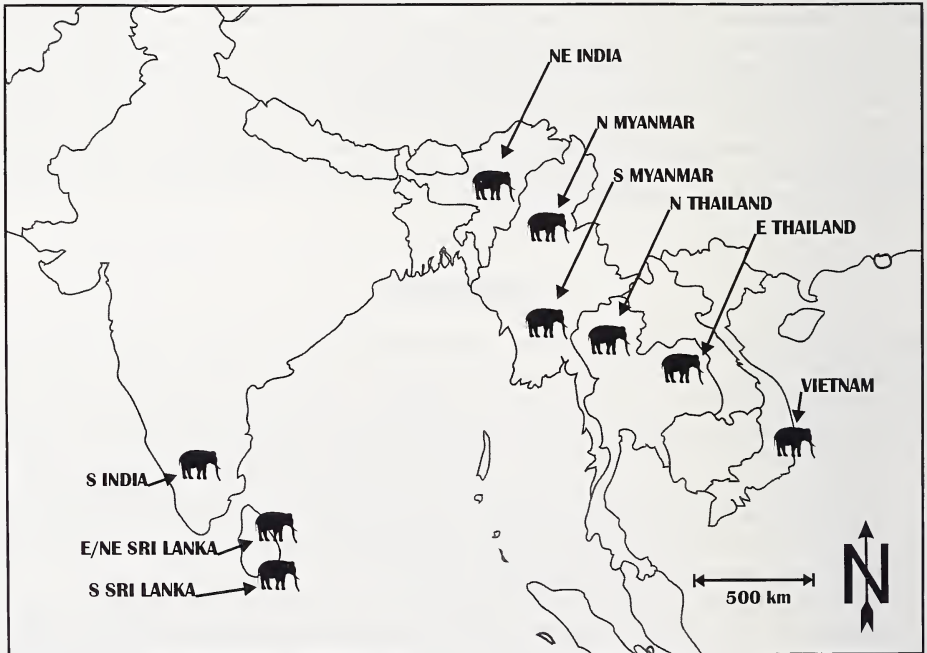


Fig. 1. Geographic origin of Asian elephants included in the present study.

Upper Chidwan on the Myanmar side; 4. Southern Myanmar and northern Thailand, since a considerable movement of elephants occurs between those areas; 5. Eastern Thailand and Vietnam, with a continuous population until recent times (cf. SUKUMAR 1992).

Amplification and sequencing of the mitochondrial cytochrome b gene

An about 480 bp part of the mitochondrial cytochrome b gene was amplified via the Polymerase Chain Reaction (PCR) using the primers L 14724 and H 15149 (IRWIN et al. 1991). Assay conditions were 2.5 U Taq-polymerase (Perkin Elmer), 0.2 μ M of each primer, and 50 μ M of each nucleotide ATP, CTP, GTP, and TTP in a reaction volume of 75 μ l. The bulb end of a single hair was set directly into the reaction solution, without any prior treatment. After an initial denaturation step at 97 °C for 15 min, 39 cycles were carried out with denaturation at 93 °C for 1:30 min, annealing at 53 °C for 1:15 min, and extension at 72 °C for 1:30 min, followed by a final extension at 72 °C for 3 min.

The PCR-products were purified using a commercial kit (Qiagen) and cycle-sequenced using the SequiTherm Cycle Sequencing Kit (Epicentre) and the Digoxigenin-labelled oligonucleotide 5'-DIG-GCTTGATGAAAAACCATCGTTG-3' for 30 cycles, each with a denaturation at 95 °C for 30 s, an annealing at 52.7 °C for 30 s, and an extension at 70 °C for 1 min. Samples were run on a direct blot sequencing device (RICHTERICH et al. 1989). After blotting onto a nylon membrane, sequences were detected using an anti-Digoxigenin/Alkaline phosphatase conjugate (Boehringer) and the chemiluminescent substrate CDP-Star (Tropix), following manufacturer's instructions.

Data analysis

Based on 335 bp scored sequence of the cytochrome b gene, mitochondrial haplotypes were defined and compared in terms of pairwise nucleotide divergence (NEI and JIN 1989), using the Neighbor-Joining method (SAITOU and NEI 1987) and the PHYLIP 3.5c computer package (FELSENSTEIN 1993). Published sequences of the African elephant (*Loxodonta africana*, IRWIN et al. 1991), and of the Siberian woolly mammoth (*Mammuthus primigenius*, HAGELBERG et al. 1994) served as outgroups. A median graph of the relationships between mitochondrial haplotypes was determined according to BANDELT (1992). Mean nucleotide diversities within and between geographic subsets of the analysed samples were calculated (cf. QUINN and WHITE 1987). Standard errors of these values were estimated by bootstrap resampling of nucleotides for 1000 times. Haplotype diversities at different localities were calculated according to NEI and TAJIMA (1981).

Results

Compared with the corresponding Cyt b sequence in the African elephant, the sequence of the Asian elephant differed by 18 to 25 nucleotide substitutions. Excluding the first 95 nucleotides not scored by HAGELBERG et al. (1994), Asian elephant differed from both the African elephant and the Siberian woolly mammoth by 8 to 15 nucleotide substitutions. All but one substitutions were transitions. The only transversion (A \leftrightarrow C at position 129) found separated the African elephant from the other two species (Fig. 2). In the 53 Asian elephants analysed, a total of eight mitochondrial haplotypes were found, differing from one another by one to six nucleotides at altogether seven polymorphic sites (Fig. 3). All seven mutations were transitions at the third codon position, without an effect on the amino acid composition of the protein.

Using the Neighbor-Joining procedure, haplotypes separated out by nucleotide divergence into two major clusters A and B, consisting of MAX I, II, III, V, VIII, and of MAX IV, VI, VII, respectively (Fig. 4). Each cluster contained one of the two most common mitochondrial haplotypes, MAX V (found in 18 specimens) and MAX VI (found in 12 specimens), respectively. Within each cluster, all other haplotypes can be derived by one to two transitions from these frequent haplotypes (Fig. 5). While the frequent haplotypes MAX V and MAX VI were distributed over the whole study area, some of the other hap-

lotypes (e.g. MAX I, MAX IV, MAX VIII) were found only in particular study units (Tab. 1).

10	20	30	40	50	60	
ATGACCGACATTCGAAAATCTCACCCCTTACTTAAAATCATCAATAAATCCTTCATTGAT						<i>L. a.</i>
.....C...CC.....C.GT.....C.....						<i>E. m.</i>
70	80	90	100	110	120	
CTACCTACCCCATCCAACATATCAACATGATGAAATTCGGCTCACTACTAGGAGCATGC						<i>L. a.</i>
.....						<i>M. p. 1</i>
.....						<i>M. p. 2</i>
.....C.....T.....C.....						<i>E. m.</i>
			T		G	
130	140	150	160	170	180	
CTAATTACACAAATCCTAACAGGATTATTCCTAGCCATACATTATACACCCGACACAATA						<i>L. a.</i>
.....C..G.....T.....						<i>M. p. 1</i>
.....C.....G.....T.....T.....T.....						<i>M. p. 2</i>
.....C.....C.....T.....						<i>E. m.</i>
190	200	210	220	230	240	
ACTGCATTTTCATCTATATCCCATATTTGCCGAGATGTAAACTACGGCTGAATTATTCGA						<i>L. a.</i>
.....C.....						<i>M. p. 1</i>
.....C.....C.....T.....						<i>M. p. 2</i>
.....C.....C..C.....						<i>E. m.</i>
	C					
250	260	270	280	290	300	
CAACTACACTCAAACGGAGCATCCATTTTCTTCTCTGCCTATACACACATTGGACGA						<i>L. a.</i>
.....T.....						<i>M. p. 1</i>
.....T.....						<i>M. p. 2</i>
.....T.....						<i>E. m.</i>
	G		C T			
310	320	330				
AACATCTACTATGGGTCCTACTATACTCGGAAACTTGAA						<i>L. a.</i>
.....C.....						<i>M. p. 1</i>
.....C.....						<i>M. p. 2</i>
.....A.....A.....C.....						<i>E. m.</i>
		T				

Fig. 2. Nucleotide sequences of part of the Cyt b gene in Asian elephants (*E. m.*) in comparison to published sequences from one African elephant (*L. a.*, IRWIN et al. 1991), and two Siberian woolly mammoths (*M. p. 1*, *M. p. 2*, HAGELBERG et al. 1994). Additional capital letters indicate polymorphic sites in Asian elephant.

	1112223
	0194671
	2756706
MAX I	TGTGCTT
MAX II	C.C...C
MAX III	C...C.
MAX IV	CA.A..C
MAX V	C.....
MAX VI	CA.ATCC
MAX VII	CA.A.CC
MAX VIII	C.....C

Fig. 3. Sequence polymorphisms in the different haplotypes of Asian elephants. Position numbers are indicated by three vertical digits.

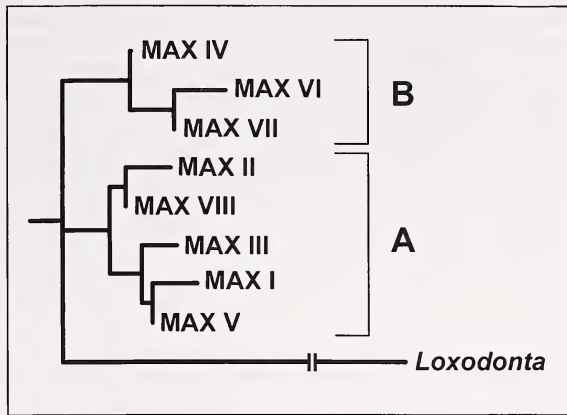


Fig. 4. Sequence divergence among haplotypes of Asian elephant (Neighbor-joining tree, based on nucleotide divergence according to NEI and JIN 1989). The African elephant (*Loxodonta*) was used as an outgroup (according to our results the transition/transversion ratio was estimated to be 20/1). A and B refer to clusters of similar haplotypes as defined in Figs. 2 and 3.

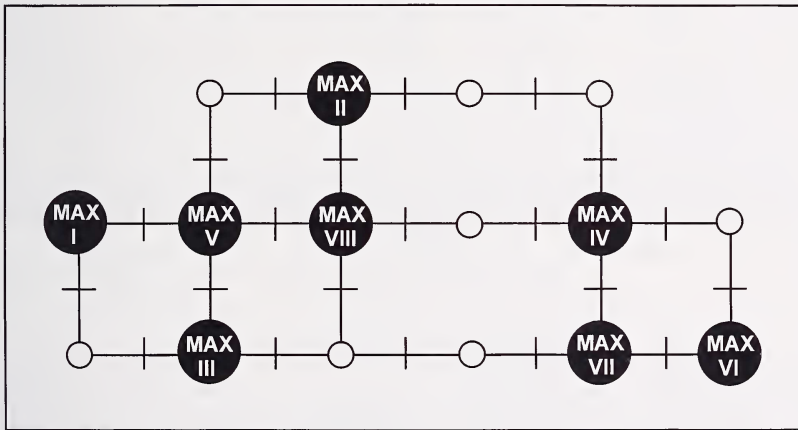


Fig. 5. Median graph according to BANDELTI (1992), showing relationships among haplotypes in terms of nucleotide substitutions (small bars). Supposed transient haplotypes not found in this study are indicated by circles (see Figs. 2 and 3 for definition of haplotypes).

Nucleotide diversity and haplotype diversity, characterizing genetic variability within study units, are given in table 1. Haplotype diversity was highest in Sri Lanka and in northeastern India/northern Myanmar. The latter area also showed the highest nucleotide diversity. Genetic differentiation among study units in terms of nucleotide diversity is given in table 2.

Table 1. Geographic distribution of cytochrome b haplotypes (MAX I–MAX VIII) found in Asian elephant. Letters in parentheses refer to major groups of haplotypes as defined in Fig. 4. Total T = total number of haplotypes found in the respective samples. S SL = southern Sri Lanka, NE SL = northern and eastern Sri Lanka, S In (NE In) = southern (northeastern) India, N My (S My) = northern (southern) Myanmar, N Tha (S Tha) = northern (southern) Thailand, Viet = Vietnam. Numbers in parentheses refer to study units created by pooling of single samples. h = haplotype diversity, π = nucleotide diversity (in per cent) for the respective study units (standard error in parentheses).

Type MAX	S SL (1) n = 2	NE SL (1) n = 12	S In (2) n = 9	NE In (3) n = 3	N My (3) n = 8	S My (4) n = 3	N Tha (4) n = 5	E Tha (5) n = 6	Viet (5) n = 5
I (A)		2							
II (A)		3			4				
III (A)		1					3	1	1
IV (B)		1							
V (A)	1	4	5	1	1	3		2	1
VI (B)	1	1	2	1	1			3	3
VII (B)				1	2		2		
VIII (A)			2						
total T:	2	6	3	3	4	1	2	3	3
h		0.84	0.67		0.80		0.75		0.65
π		0.91 (0.36)	0.77 (0.33)		1.20 (0.47)		0.75 (0.33)		0.94 (0.37)

Table 2. Pairwise nucleotide diversity (in per cent, above diagonal) and net nucleotide diversity (in per cent, below diagonal) between study units (1–5, see Table 1) of Asian elephant (standard error in parentheses).

	1	2	3	4	5
1	–	0.90 (0.37)	1.18 (0.47)	0.88 (0.37)	1.19 (0.45)
2	0.06 (0.03)	–	1.21 (0.45)	0.90 (0.40)	1.06 (0.43)
3	0.13 (0.06)	0.22 (0.09)	–	1.17 (0.47)	1.38 (0.55)
4	0.06 (0.03)	0.18 (0.09)	0.22 (0.11)	–	1.26 (0.53)
5	0.28 (0.10)	0.21 (0.09)	0.31 (0.12)	0.41 (0.17)	–

Discussion

Cytochrome b variation in the Asian elephant

Data available on sequence variation of the mitochondrial cytochrome b gene have proved to be inconsistent with respect to genetic variation within mammalian populations (see e.g. SHIELDS and KOCHER 1991; RANDI et al. 1994 for bears; PERRY et al. 1995 for seals; CARR and HUGHES 1993 for deer; WAYNE and JENKS 1991; GIRMAN et al. 1993 for wild canids).

Using restriction enzymes for cutting a 2,450 bp fragment of mtDNA, GEORGIADIS et al. (1994) detected considerable genetic variation within and among populations of the African elephant (*Loxodonta africana*). This result was interpreted to indicate a large long-term effective population size and high gene flow between subpopulations. Exhibiting a total of 7 variable sites and considerable haplotype diversity within each of the local populations studied, also the Asian elephant, assumed to originate from Africa and to have invaded southern and southeastern Asia during the Pliocene (cf. HAYNES 1991), can be considered at least moderately polymorphic. Despite a remarkable decline and increasing isolation of local Asian elephant populations in historical times due to human exploitation (KURT et al. 1995), the observed variation may indicate the ancient effective population size to have been large.

Relationships among haplotypes in comparison with their geographic distribution

Haplotype MAX VI was previously described in six Asian elephants without mentioning their geographic origin (HAGELBERG et al. 1994). This haplotype is the one most closely related to those of both the African elephant and the Siberian woolly mammoth, and taking also into account its widespread geographic distribution it must be considered a very basic one. According to their geographic distribution the same holds for haplotype MAX V and, although being absent or rare in the central and easternmost part of our study area, respectively, for haplotypes MAX III and MAX II. Haplotype MAX I, apparently confined to Sri Lanka may have locally arisen through a new mutation, which is in agreement with its marginal position in the median graph. However, due to their more central position in the median graph, the other haplotypes found only in single study units (MAX IV, MAX VIII) or in a group of adjacent study units (MAX VII) may rather represent relics of a formerly more widespread polymorphism. Altogether, the disjunct distribution of many haplotypes suggests that they had already been present at the time when elephant colonized southern and southeastern Asia during the Pliocene (c. f. HAYNES 1991). This argument is supported by the presence of several well differentiated haplotypes at Cyt b, a generally rather conservative gene with respect to variation at the population level (cf. AVISE 1994). The alternative hypothesis, a segregation of haplotypes MAX IV and MAX VIII in the Indian subcontinent, and of haplotype MAX VII in Southeastern Asia, respectively, from a basic set consisting of haplotypes MAX VI, MAX V, MAX II, and MAX III in the original founder population would imply a considerable extent of homoplasy, which is unlikely to have arisen at the population level.

Genetic diversity within populations

The assumption of local losses of Cyt b haplotypes in Asian elephant is supported by levels of nucleotide and haplotype diversity found in extant populations. However, it is interesting to note that, in a comparison of populations studied, estimates of nucleotide diversity do not correspond with the respective levels of haplotype diversity. This can be explained by both the number of haplotypes and the extent of nucleotide divergence among haplotypes being involved in the calculation of nucleotide diversity. The largest number of haplotypes is found in Sri Lanka, but with the exception of two animals with haplotype MAX VI all of them belong to group A. Hence, the comparatively high nucleotide diversity in that area mainly reflects a large number of haplotypes present. In northern India/northern Myanmar the number of haplotypes found is smaller than in Sri Lanka, but the proportions of haplotypes belonging to group A and B, respectively, the two being separated by two mutation steps, are more similar than in the previous case. In eastern Thailand/Vietnam the high nucleotide diversity is almost exclusively due to a large nucleotide divergence among only a few haplotypes. As a methodological consequence

we suggest that both haplotype diversity and nucleotide diversity are essential for a proper characterization of genetic variability in populations.

The most striking result with respect to levels of genetic variability is the high haplotype diversity present on the island of Sri Lanka as compared to the respective values found in most areas of the Indian subcontinent and in southeastern Asia. During the last five centuries, nowhere in the range of the Asian elephant has capturing and killing by Mogul and colonial forces been carried out as extensively as in the area between southern and northeastern India. There numbers of elephants have declined from at least 375,000 at 330 BC to less than 20,000 at present (i. e. to about 5 per cent of the original population). In southern Myanmar and in Thailand large numbers of elephants had been caught for timber extraction between the late 18th and the early 20th century (EVANS 1910; STRACEY 1963). In addition it must be stressed that, due to habitat destruction by man, on the Indian subcontinent the remaining elephant population is subdivided into a number of mostly small isolates, which are exposed to genetic drift (KURT 1992, KURT et al. 1995). A similar situation prevails in the region from southern Myanmar to Vietnam (SANTHAPILLAI and JACKSON 1990).

By contrast, in Sri Lanka still some 20 per cent of the original elephant population of about 12,000 individuals are present, and human encroachment hardly prevented migration across the whole island until the middle of this century (KURT 1992). Also in northeastern India and northern Myanmar, harbouring the second highest haplotype diversity, human impact on population size of elephant had been comparatively low. Altogether, the different levels of genetic variation found in populations of Asian elephant are in good agreement with records on population histories.

Genetic diversity among populations

Although there seems to be a tendency of haplotypes belonging to group A to decrease from the western to the eastern part of our study area, haplotype frequencies and estimates of net nucleotide diversity among samples do not correspond very well with the geographic distribution of the local populations studied. A certain bias due to limited sample sizes of individuals cannot be excluded, but in general this result seems to reflect a considerable extent of genetic drift caused by human impact on population size and structure.

Implications for systematics and conservation of Asian elephant

Estimates of net nucleotide diversity among populations do not reveal a major separation of the Sri Lanka population from all other study units. Thus, our data do not provide support for subspecies distinction between elephants on Sri Lanka and on the Asian mainland, which is in accordance with the electrophoretic data of HARTL et al. (1995). In the light of our present results the apparently fixed difference at the Sod-2 locus reported by NOZAWA and SHOTAKE (1990) can be explained by short-term drift processes in local populations rather than by a long-term divergence between subspecies.

Regarding breeding of Asian elephants in zoos differentiation among wild-living populations, especially between those of Sri Lanka and southern India, formerly believed to represent different subspecies, seems to be low enough to justify cross-breeding of parents of geographically different origin. Future management of wild-living and captive populations in Asia should follow the traditional Sri Lankan practice, where the off-take from the wild-living population for maintaining considerable captive stock has been as high as necessary but low enough for preserving the highest level of genetic variability as yet known (see also KURT et al. 1995).

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Zusammenfassung

Populationsgenetik und Systematik des Asiatischen Elefanten (Elephas maximus): Eine Studie auf der Grundlage von Sequenzvariation am Cyt-b-Gen von PCR-amplifizierter Mitochondrien-DNA aus Haarwurzeln

Um die genetische Variabilität und Differenzierung bei Asiatischen Elefanten (*Elephas maximus*) zu charakterisieren, wurde bei insgesamt 53 Individuen aus Sri Lanka, dem Süden und Nordosten Indiens, dem Norden und Süden von Myanmar (Burma), dem Norden und Osten von Thailand sowie aus Vietnam die Sequenzvariation am mitochondrialen Cytochrom-b-Gen untersucht. Ein ca. 480 bp langer Abschnitt des Gens wurde mittels PCR aus Haarwurzeln amplifiziert und im Thermocycler direkt sequenziert. Nach Auftrennung in einer Direkt-Blot-Sequenzieranlage wurden 335 bp analysiert. Sieben Sequenzpositionen waren polymorph, und acht Haplotypen wurden definiert, die sich durch eine bis sechs Transitionen an der dritten Codon-Position unterschieden. Die Haplotypen wichen im Vergleich zu publizierten Sequenzen an mindestens 18 Positionen vom Afrikanischen Elefanten (*Loxodonta africana*) und an mindestens 8 Positionen vom Mammuth (*Mammuthus primigenius*) ab. Entsprechend ihrer Nukleotiddivergenz ließen sich die Haplotypen zwei Gruppen zuordnen, die sich in ihrer geographischen Verbreitung unterschieden. Die Divergenz zwischen Elefanten von Sri Lanka und dem asiatischen Festland, die bisher als eigene Unterarten beschrieben worden waren, war zum Teil kleiner als zwischen Elefantenbeständen auf dem asiatischen Festland. Die untersuchten Bestände zeigten deutliche Unterschiede hinsichtlich der Haplotypen- und Nukleotiddiversität. Die phylogeographischen Muster der Haplotypen legen nahe, daß die Asiatischen Elefanten zur Zeit ihrer pliozänen Einwanderung nach Süd- und Südostasien eine zusammenhängende Population gebildet haben. Unter Berücksichtigung der jeweiligen Bestandesgeschichte läßt sich die gegenwärtige geographische Differenzierung als Folge einer unterschiedlich starken menschlichen Einflußnahme auf die Bestandesgrößen und -strukturen interpretieren.

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